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Morphological Divergence, Reproductive Isolating Mechanism, and Molecular Phylogenetic Relationships Among Indonesia, Malaysia, and Japan Populations of the *Fejervarya limnocharis* Complex (Anura, Ranidae)

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In order to elucidate the taxonomic status of the *Fejervarya limnocharis* complex relative to Malaysia and Japan populations, morphological observations and molecular phylogenetic analysis were carried out using three populations from Indonesia (type locality), Malaysia, and Japan. In addition, we conducted histological and spermatogenic observations using hybrids among these populations. Principal component and cluster analyses demonstrated that these populations could be clearly separated from one another. Abnormal testes were found in the hybrids between the Japan and Indonesia populations and between the Japan and Malaysia populations, but testes of the controls and hybrids between the Malaysia and Indonesia populations were quite normal. The mean number of univalents per cell was 5.42, 4.58, and 0.20 in hybrids between the Indonesia and Japan populations, Malaysia and Japan populations, and Indonesia and Malaysia populations, respectively. Sequence divergences in 16S rRNA and Cyt *b* genes were 0–0.4% ($\bar{x}$=0.2%) and 0.3–1.5% ($\bar{x}$=1.0%), respectively, between the Malaysia and Indonesia populations, and 2.4–2.6% ($\bar{x}$=2.5%) and 11.0–12.0% ($\bar{x}$=11.5%) between the Japan population and *F. limnocharis* complex, including the Malaysia and Indonesia populations and *F. multistriata* from China. This study indicated that the Malaysia population and *F. multistriata* from China should be designated as a subspecies of topotypic *F. limnocharis,* and that the Japan population should be regarded as a distinct species.

Key words: morphology, spermatogenesis, sequence divergence, molecular phylogeny, Asia, *Fejervarya limnocharis*, species complex, speciation

INTRODUCTION

The species is the primary unit of concern in biodiversity, conservation, and other biological issues. More than 25 species concepts are recognized in the literature (reviewed by de Queiroz, 1998; Coyne and Orr, 2004), each with its own limitations (Hey, 2001). These authors pointed out that reproductive isolation is an important component of all lineages based on the phylogenetic species concept. Hanken (1999) argued that the biological species concept emphasizes the degree of actual or potential reproductive isolation as the predominant criterion for assessing taxonomic identity. Bradley and Baker (2001) mentioned that in the process of determining the validity of putative species, systematists generally rely on indirect information in the form of the same, characteristic systems, e.g., variation in size and shape of morphologic characteristics, cytogenetics, allozymes, and DNA sequences. Consequently, de Queiroz (1998) and Hey (2006) developed the general lineage species concept, stressing species as an independent evolutionary lineage diagnosed by multiple criteria. Multiple criteria are very useful for determining species in species

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complexes, e.g., sympatric and allopatric species. Application of multiple criteria has been carried out for elucidating the species complex in *Philautus* (Ranidae, Rhacophorinae) from Sri Lanka (Meegaskumbura and Manamendra-Arachchi, 2005), in *Discoglossus* from the western Mediterranean (Zangari *et al*., 2006), in neotropical hylid frogs (Lougheed *et al*., 2006), and in several frogs from Malagasy (Vences *et al*., 2003).

Fejervarya limnocharis is a species complex of frogs (Toda *et al.*, 1998; Iskandar and Colijn, 2000) widely distributed in Asia from Pakistan to Japan and Indonesia (Iskandar, 1998; Iskandar and Colijn, 2000; Dubois and Ohler, 2000). It probably consists of several species or subspecies over all of Asia (Dharne *et al*., 2004; Djong *et al*., 2007; Sumida *et al*., 2007). Recently, analyses using non-morphological characteristics such as mating calls, allozymes, and mitochondrial DNA revealed that *F. limnocharis* is a composite of several different species (Dubois, 1975, 1992; Dutta, 1997; Toda *et al*., 1997, 1998; Veith *et al.*, 2001). Dubois (1992) proposed that the whole group was composed of at least 15 species and probably many more in South India. Dutta (1997) reported nine nominal species in India. Toda *et al.* (1998) suggested the presence of at least four species in Southeast Asia, based on allozyme data. Djong *et al.* (2007) found that, based on allozyme analyses and crossing experiments, the *F. limnocharis* complex can be divided into two groups, the *F. limnocharis* group and the *F. iskandari* group. The *F. limnocharis* group consists of topotypic *F. limnocharis* and the Malaysia and Japan populations, while the *F. iskandari* group consists of topotypic *F. iskandari* and populations from Thailand and Bangladesh. The *F. limnocharis* group might comprise several species or subspecies.

In the present study, we studied the morphological divergence, reproductive isolating mechanisms, and molecular phylogenetic relationships among Indonesia (type locality), Malaysia, and Japan populations, to elucidate the taxonomic status of the Malaysia and Japan populations by using multiple criteria and by comparing them with topotypic *F. limnocharis*.

MATERIALS AND METHODS

Morphometry

A total of 45 mature specimens consisting of 28 males and 17 females were used for morphological observations. These were from three populations of the *Fejervarya limnocharis* group: Higasihiroshima (Japan), Kuala Lumpur (Malaysia), and Bogor (Java, Indonesia, type locality). These specimens were deposited in the Institute for Amphibian Biology, Hiroshima University (IABHU). Thirty-one characters were measured with calipers to the nearest 0.1 mm; these characters were as follows, with abbreviations in parentheses: snout-vent length (SVL), head length (HL), head width (HW), snout-tympanum length (STL), mouth angle-snout length (MSL), distance from nostril to tip of snout (NS), distance from front of eye to tip of snout (SL), nostril-tympanum length (NTL), distance from front of eye to nostril (EN), tympanum-eye distance (TEL), tympanum diameter (TD), distance from back of mandible to nostril (MN), distance from back of mandible to front of eye (MFE), distance from back of mandible to back of eye (MBE), internarial space (IN), eye length (EL), interorbital distance (IOD), maximum width of upper eyelids (UEW), hand length (HAL), forearm length (FAL), lower arm length (LAL), hindlimb length (HLL), thigh length (THIGHL), tibia length (TL), foot length (FOL), length of tarsus and foot (TFOL), third finger length (3FL), first finger length (1FL), fourth toe length (4TL), length of inner metatarsal tubercle (IMTL), inner toe length (ITL).

To standardize different over-all body size among specimens, all measurements were divided by snout-vent length (SVL) and are shown as percentages. The data were transformed into $\log_{10}$ values before cluster and principal component analyses (PCA) using MVSP 3.1 software. Morphological variation among populations was examined by the nonparametric Kruskall-Wallis test, and differences between populations were tested using the nonparametric Mann-Whitney U test at a significance level of 5% using SPSS statistics software for personal computers.

Histology and observations of spermatogenesis

Testes of mature hybrids among three populations and the controls produced by Djong *et al.* (2007) were used for histological and spermatogenic observations. One testis was fixed in Navashin's solution, sectioned at 10 μm, and stained with Heidenhain's iron hematoxylin for histological observation, while the other was used to make chromosome preparations. Meiotic chromosomes were prepared according to the technique described by Schmid *et al*. (1979) with a slight modification. The chromosomes were stained with a 4% Giemsa solution for 15 min. Chromosome analyses were carried out using only diploid cells at diakinesis and metaphase of the first reduction division, when bivalent and univalent chromosomes could be easily distinguished from each other.

DNA extraction, PCR, and sequencing

The specimens used for molecular analysis are listed in Table 1. Total genomic DNA was extracted from the clipped toes of each frog using a DNA extraction kit (DNeasy® Tissue Kit, QIAGEN). Two sets of primers, F51 and R51 (Sumida *et al.,* 2002), and Fow 1-1 and Rev-1, were used for amplification and sequencing of the 5' portion of the 16S rRNA and Cyt *b* genes corresponding to positions 6189–6761 and 16662–17491, respectively, in *Fejervarya limnocharis* (probably *F. multistriata*) (Liu *et al.,* 2005). The primer sequences were F51 (5'-CCC GCC TGT TTA CCA AAA ACA T-3'), R51 (5'-GGT CTG AAC TCA GAT CAC GTA-3'), Fow 1-1 (5'-ACM GGH YTM TTY YTR GC ATR CAY TA-3') and Rev-1 (5'-TAD GCR AAW AGR AAR TAY CAY TCN GG-3'). PCR mixtures were prepared with the TaKaRa Ex Taq™ Kit according to the manufacturer's protocol. The 16S rRNA and Cyt *b* genes were amplified by 35 cycles of 10 sec at 98°C, 30 sec at 50°C, and 1 min 20 sec at 72°C. The PCR products were sequenced with an automated DNA sequencer (3100-Avant, ABI) with the BigDye® Terminator Cycle Sequencing Kit (ABI). The resultant sequences were deposited in the DDBJ database (accession nos. AB296085–AB296101). Nucleotide sequences were analyzed using DNASIS (Ver.3.2, Hitachi Software Engineering) and Clustal W (Thompson *et al.*, 1994).

Phylogenetic analysis

Nucleotide sequences of the 16S rRNA and Cyt *b* genes from nine and 12 specimens of the *F. limnocharis* group, respectively, were aligned using Clustal W with ambiguous sites manually eliminated. Phylogenetic analyses were performed by the maximum likelihood (ML), neighbor-joining (NJ), and maximum-parsimony (MP) methods, and sequence divergence among haplotypes was calculated as uncorrected *p*-values using PAUP* Ver.4.10b (Swofford, 2002). *Limnonectes fujianensis* was used as an outgroup (Accession No. AY974191, Nie *et al*., unpublished). The ML and NJ analyses were carried out using substitution models and parameters estimated by MODELTEST Ver. 3.06 (Posada and Crandall, 1988). The MP tree was constructed under a heuristic search with ten replicates, using simple sequence addition and tree bisection-reconnection (TBR). This tree was then used as a starting tree for ML analysis. The reliability of the resultant trees was evaluated by bootstrap (BP) percentages based on analyses of 1,000 pseudoreplicates.

Table 1. Specimens of the *F. limnocharis* complex used in the present molecular study and haplotypes of nucleotide sequences of the Cyt *b* and 16S rRNA genes.

Species	Locality	Individual number	Haplotype	Accession number	
				Cyt *b*	16S rRNA
Fejervarya iskandari	Cianjur, Java, Indonesia	1	iska-cian	AB296085	AB277303[1)]
F. iskandari	Malingping, Java, Indonesia	1	iska-malin	AB296086	–
F. limnocharis	Bogor, Java, Indonesia	1	limn-bogo	AB296087	AB277302[1)]
F. limnocharis	Malingping, Java, Indonesia	1	limn-malin	AB296088	AB277292[1)]
F. limnocharis	University of Malaya	3	limn-kual-1	AB296089	AB296097
	Campus, Kuala Lumpur,		limn-kual-2	AB296090	AB296098
	Malaysia		limn-kual-3	AB296091	–
F. limnocharis	Kota Kinabalu, Saba,	2	limn-sara-1	AB296092	AB296099
	Malaysia		limn-sara-2	AB296093	AB296100
F. multistriata	Hainan, China	1	mult	AB296094	AB296101
F. limnocharis	Higashihiroshima, Japan	1	japo-higa	AB296095	AB070732[2)]
F. limnocharis	Hiroshima, Japan	1	japo-hiro	AB296096	–
Limnonectes fujianensis	China	1	–	AY974191[3)]	AY974191[3)]

[1)] Data from Kotaki *et al*. (2008)
[2)] Data from Sumida *et al.* (2002)
[3)] Data from Nie *et al*. (unpublished)

RESULTS

Morphometry

UPGMA dendrograms based on Euclidean distance showed that the three populations could be divided into two clusters, the Japan population and the Malaysia and Indonesia populations, in both males and females. The second cluster could be divided into two subclusters, the Malaysia and Indonesia populations (Fig. 1A, B).

Comparison of adult specimens among the three populations using the Kruskall-Wallis test showed significant differences among them in 28 morphometric parameters in males, and at 23 in females (Tables 2 and 3). Based on the Mann-Whitney *U* test to compare the differentiation between genetically distinct samples, the Malaysia and Indonesia populations were significantly different in males in only nine parameters, and in females in ten parameters (Tables 2 and 3). On the other hand, 24 parameters were significantly different in males between the Indonesia and Japan populations, and 12 parameters in females (Tables 2 and 3). The Malaysia and Japan populations had 26 significantly different parameters in males and 19 in females (Tables 2 and 3).

Principal component analysis (PCA) based on the 31 $\log_{10}$-transformed morphometric distances showed that the three populations are clearly differentiated both in males and females (Fig 2A, B). Two components were extracted with eigenvalues >1 that explained 46.35% and 40.86% (first component) and 16.47% and 17.69% (second component) of all morphometric variation in males and females, respectively (Table 4). Characters describing the forelimbs (HAL and FAL) and hindlimbs (HLL, THIGHL, TL, FOL, TFOL and 4TL) dominated, with high positive loading in the first component (PC1) in both males and females. In the second component (PC2), characters describing head size (MFE, HL and HW) dominated in males, with high positive loading, but characters describing metatarsal tubercle size (IMTL) and head size (HL and HW) dominated with high negative loading in females. Thus, PC1 represented differences in hand and leg proportion, and PC2 represented different proportions in head shape.

Histological observations

Cross-sections of seminiferous tubules of the testes in hybrids among the three populations and the controls are shown in Fig. 3. In the control Japan and Malaysia populations, the testes were completely normal and the seminiferous tubules were filled with dense bundles of normal spermatozoa (Fig. 3A, B), and the same condition was found in hybrids between Malaysia and Indonesia populations (Fig. 3E). In the hybrids between Japan and Indonesia populations and between Japan and Malaysia populations, the testes showed some abnormality: the bundles of spermatozoa were small and coarse, and sparsely distributed, abnormal spermatozoa and pycnotic nuclei were observed (Fig. 3C, D).

Spermatogenesis

Spermatocytes at the first meiosis and chromosome complements in the hybrids among the three populations and the controls are shown in Fig. 4A–F. The diploid number of chromosomes in the *F. limnocharis* group was 26, and in the normal first meiotic division, chromosomes consisted of 13 bivalents. In the control Japan and Malaysia populations, most meiotic spreads comprised 13 ring-shaped bivalents, five of them large and eight small (Fig. 4A), and some spreads contained several rod-shaped bivalents in addition to the ring-shaped bivalents. Hybrids among the three populations showed several variations in the number of ring- and rod-shaped bivalents and in the number of univalents (Fig. 4B–H).

The number and frequency of meiotic spreads differing in number of univalents in male hybrids among the three populations of the *F. limnocharis* group and the controls are shown in Table 5 and Fig. 5. In the control Japan and Malaysia populations, all meiotic spreads contained 13 bivalents. In hybrids between Japan and Indonesia popula-

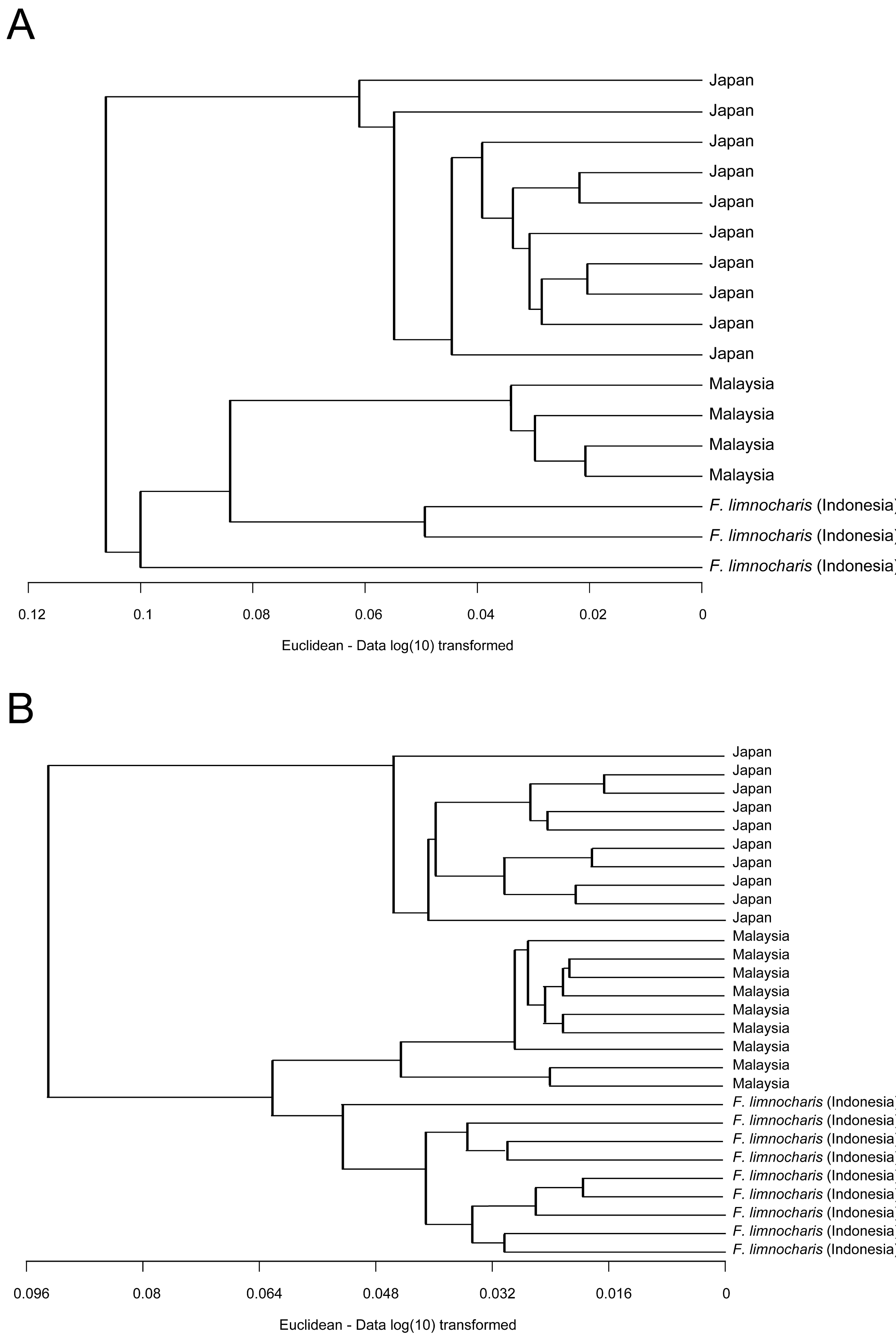

Fig. 1. UPGMA dendrograms based on morphological characters of the *F. limnocharis* group. (A) Females. (B) Males.

Table 2. Comparison of adult males of the *F. limnocharis* group by Kruskall-Wallis and Mann-Whitney U tests of snout-vent length (SVL) and of ratios of measurements from different populations. For each sample, minimum and maximum values, mean and standard deviation are given. df, degree of freedom; n, sample size; p, probability; U, Mann-Whitney U; *, significance level $p \leq 0.05$; ns, not significant. See the Materials and Methods for abbreviations of the morphometric measurement.

Morphometric measurement or ratio	*F. limnocharis* (Indonesia) n=9	Malaysia n=9	Japan n=10	Kruskall-Wallis test df=2 significance level $p \leq 0.05$	Mann-Whitney U test significance level $p \leq 0.05$		
					Indonesia-Malaysia	Indonesia-Japan	Malaysia-Japan
	39.0±3.6	38.1±2.0	37.7±1.2	x^2=0.504	U=38	U=36.5	U=39.5
SVL	34.7–44.8	35.5–41.7	36.1–39.5	p=0.777$_{ns}$	p=0.825$_{ns}$	p=0.487$_{ns}$	p=0.653$_{ns}$
	46.2±1.9	39.7±0.8	40.0±1.6	x^2=17.813	U=0	U=0	U=39.5
HL/SVL	44.0–49.6	38.8–40.6	38.0–42.7	p=0.000*	p=0.000*	p=0.000*	p=0.653$_{ns}$
	37.8±1.3	34.0±0.8	37.9±1.4	x^2=17.752	U=0	U=42	U=0
HW/SVL	36.1–40.2	32.9–35.0	36.2–40.2	p=0.000*	p=0.000*	p=0.806$_{ns}$	p=0.000*
	30.6±1.1	30.7±0.6	28.6±1.3	x^2=12.919	U=37	U=10.5	U=5
STL/SVL	29.0–32.4	29.6–31.7	26.8–30.7	p=0.002*	p=0.756$_{ns}$	p=0.005*	p=0.001*
	35.6±1.4	34.9±1.3	33.6±1.6	x^2=9.297	U=24.5	U=14.5	U=15
MSL/SVL	33.2–37.8	34.0–38.1	31.4–37.4	p=0.010*	p=0.157$_{ns}$	p=0.013*	p=0.0134*
	8.2±0.7	8.2±0.7	9.2±0.9	x^2=7.509	U=38.5	U=15	U=18
NS/SVL	6.9–9.1	7.6–9.1	7.3–10.2	p=0.023*	p=0.859$_{ns}$	p=0.014*	p=0.027*
	15.7±0.8	17.3±0.7	14.9±0.8	x^2=17.693	U=4	U=23	U0.5
SL/SVL	14.5–7.0	16.1–18.3	13.5–16.1	p=0.000*	p=0.001*	p=0.072$_{ns}$	p=0.000*
	24.0±0.7	24.7±1.0	22.3±1.1	x^2=14.186	U=30	U=8.5	U=4.5
NTL/SVL	23.1–24.9	23.5–26.1	21.2–23.8	p=0.001*	p=0.352$_{ns}$	p=0.003*	p=0.001*
	9.7±0.5	10.2±1.0	7.5±0.5	x^2=19.300	U=22	U=0	U=1
EN/SVL	8.9–10.2	8.5–11.5	6.9–8.6	p=0.000*	p=0.102$_{ns}$	p=0.000*	p=0.000*
	4.1±0.5	4.5±0.2	4.4±0.3	x^2=5.103	U=18	U=24	U=35
TEL/SVL	3.2–4.9	4.2–4.8	4.1–4.9	p=0.078$_{ns}$	p=0.046$_{ns}$	p=0.084$_{ns}$	p=0.405$_{ns}$
	7.7±0.6	7.4±0.33	8.8±0.4	x^2=17.717	U=27	U=4.5	U=0
TD/SVL	6.7–8.6	7.0–7.9	8.5–9.7	p=0.000*	p=0.232$_{ns}$	p=0.001*	p=0.000*
	39.6±2.6	36.8±1.6	35.5±2.0	x^2=10.803	U=16.5	U=9.5	U=22.5
MN/SVL	35.3–42.4	33.8–39.8	32.8–39.9	p=0.005*	p=0.034$_{ns}$	p=0.004*	p=0.066$_{ns}$
	31.8±2.3	26.7±0.7	31.0±2.5	x^2=15.320	U=5	U=32	U=2
MFE/SVL	26.8–34.8	25.6–27.9	27.3–35.5	p=0.000*	p=0.001*	p=0.288$_{ns}$	p=0.000*
	20.2±2.6	16.2±0.7	23.2±2.4	x^2=18.882	U=3.5	U=18	U=0
MBE/SVL	16.4–24.5	15.4–17.4	19.5–26.6	p=0.000*	p=0.001*	p=0.027*	p=0.000*
	8.3±0.6	8.0±0.6	8.9±0.9	x^2=7.670	U=27.5	U=19	U=16
IN/SVL	6.9–9.1	7.3–9.1	6.6–9.8	p=0.022*	p=0.250$_{ns}$	p=0.033*	p=0.018*
	13.5±1.1	13.2±0.4	14.2±1.3	x^2=9.313	U=24	U=24.5	U=9
EL/SVL	11.3–14.8	12.5–13.5	10.9–16.0	p=0.009*	p=0.142$_{ns}$	p=0.094$_{ns}$	p=0.003*
	8.1±0.7	6.1±0.4	5.2±1.0	x^2=19.070	U=0	U=3	U=15
IOD/SVL	6.9–9.3	5.6–6.5	4.1–7.6	p=0.000*	p=0.000*	p=0.001*	p=0.014*
	14.4±0.6	14.1±0.7	15.7±1.4	x^2=11.127	U=28	U=10.5	U=11.5
UEW/SVL	13.4–15.3	13.2–15.4	12.4–17.5	p=0.004*	p=0.268$_{ns}$	p=0.005*	p=0.006*
	23.1±1.1	23.5±0.9	20.7±0.9	x^2=16.252	U=35	U=5.5	U=1
HAL/SVL	21.0–24.3	21.7–25.2	19.6–22.2	p=0.000*	p=0.626$_{ns}$	p=0.000*	p=0.000*
	23.4±1.2	23.9±0.8	21.4±1.3	x^2=12.483	U=33	U=12	U=5.5
FAL/SVL	21.5–25.0	22.5–25.4	19.7–23.7	p=0.002*	p=0.507$_{ns}$	p=0.007*	p=0.000*
	26.4±2.8	29.8±1.3	29.0±1.2	x^2=9.350	U=9.5	U=19.5	U=26.5
LAL/SVL	21.8–30.0	27.6–31.7	27.2–30.9	p=0.009*	p=0.006*	p=0.037*	p=0.1330$_{ns}$
	158.9±5.6	171.4±6.4	137.1±5.7	x^2=23.002	U=4	U=0	U=0
HLL/SVL	152.4–169.6	164.9–183.0	128.0–144.4	p=0.000*	p=0.001*	p=0.000*	p=0.000*
	50.0±1.6	49.3±3.1	42.5±1.4	x^2=19.109	U=28.5	U=0	U=0
THIGHL/SVL	47.7–52.5	46.1–55.2	39.4–43.8	p=0.000*	p=0.289$_{ns}$	p=0.000*	p=0.000*
	54.2±2.2	55.1±2.8	45.7±1.7	x^2=18.765	U=34	U=0	U=0
TL/SVL	51.2–58.1	50.5–60.2	42.9–48.5	p=0.000*	p=0.566$_{ns}$	p=0.000*	p=0.000*
	53.1±2.7	55.6±2.6	47.1±2.4	x^2=17.923	U=23	U=5	U=0
FOL/SVL	47.7–56.5	52.3–60.4	42.9–50.1	p=0.000*	p=0.122$_{ns}$	p=0.001*	p=0.000*
	77.0±4.0	80.6±3.6	65.3±2.4	x^2=19.745	U=22	U=0	U=0
TFOL/SVL	72.1–82.4	74.9–87.1	60.9–69.2	p=0.000*	p=0.102$_{ns}$	p=0.000*	p=0.000*
	12.7±0.7	12.2±0.8	10.7±0.6	x^2=16.485	U=28.5	U=2	U=5
3FL/SVL	11.8–14.0	10.7–12.9	9.9–11.9	p=0.000*	p=0.287$_{ns}$	p=0.000*	p=0.000*
	12.9±0.7	12.4±0.7	11.3±1.1	x^2=11.401	U=20.5	U=9	U=17
1FL/SVL	11.4–13.7	11.4–13.1	8.9–13.2	p=0.003*	p=0.076$_{ns}$	p=0.003*	p=0.021*
	32.0±1.6	34.0±2.2	25.7±1.6	x^2=19.597	U=23.5	U=0	U=0
4TL/SVL	29.0–33.8	31.0–37.0	23.3–28.6	p=0.000*	p=0.132$_{ns}$	p=0.000*	p=0.000*
	6.6±0.6	6.2±0.3	6.7±0.6	x^2=3.938	U=26	U=40.5	U=20.5
IMTL/SVL	6.0–7.6	5.7–6.6	5.6–7.3	p=0.140$_{ns}$	p=0.197$_{ns}$	p=0.712$_{ns}$	p=0.044*
	10.7±1.1	17.4±0.7	9.7±0.5	x^2=20.013	U=0	U=18	U=0
ITL/SVL	9.4–12.3	15.8–18.1	9.1–10.5	p=0.000*	p=0.000*	p=0.027*	p=0.000*

Table 3. Comparison of adult females of the *F. limnocharis* group by Kruskall-Wallis and Mann-Whitney *U* tests of snout-vent length (SVL) and of ratios of measurements from different populations. For each sample, minimum and maximum values, mean and standard deviation are given. df, degree of freedom; n, sample size; *p*, probability; *U*, Mann-Whitney *U*; *, significant level $p \leq 0.05$; ns, not significant. See the Material and Methods for abbreviations of the morphometric measurement.

Morphometric measurement or ratio	*F. limnocharis* (Indonesia) n=3	Malaysia n=4	Japan n=10	Kruskall-Wallis test df = 2 significance level $p \leq 0.05$	Mann-Whitney U test significance level $p \leq 0.05$		
					Indonesia-Malaysia	Indonesia-Japan	Malaysia-Japan
	45.3±7.4	47.1±0.8	42.7±1.5	x^2=6.286	*U*=4	*U*=14	*U*=0
SVL	40.4−53.8	46.3−47.9	40.1−45.2	*p*=0.043*	*p*=0.480$_{ns}$	*p*=0.866$_{ns}$	*p*=0.005*
	46.1±2.0	40.3±1.3	40.1±0.9	x^2=7.132	*U*=0	*U*=0	*U*=17
HL/SVL	44.1−48.0	38.8−41.7	38.7−41.8	*p*=0.028*	*p*=0.034*	*p*=0.011*	*p*=0.671$_{ns}$
	38.0±1.2	33.8±0.7	36.8±1.5	x^2=7.200	*U*=0	*U*=11	*U*=3
HW/SVL	37.1−39.3	32.9−34.5	33.4−38.1	*p*=0.027*	*p*=0.034*	*p*=0.496$_{ns}$	*p*=0.016*
	29.6±1.7	30.4±1.0	28.2±0.9	x^2=7.550	*U*=5.5	*U*=8	*U*=0
STL/SVL	27.7−30.9	29.4−31.8	26.6−29.3	*p*=0.023*	*p*=0.858$_{ns}$	*p*=0.236$_{ns}$	*p*=0.005*
	34.2±2.0	34.6±1.6	32.4±1.3	x^2=5.874	*U*=5	*U*=6	*U*=4.5
MSL/SVL	32.0−36.0	32.9−36.7	30.2−34.3	*p*=0.053*	*p*=0.724$_{ns}$	*p*=0.128$_{ns}$	*p*=0.0280*
	8.9±0.8	7.1±0.4	8.8±0.5	x^2=8.983	*U*=0	*U*=11	*U*=0
NS/SVL	7.9−9.4	6.5−7.4	7.8−9.4	*p*=0.011*	*p*=0.034*	*p*=0.497$_{ns}$	*p*=0.005*
	16.1±1.2	16.6±1.2	15.6±0.7	x^2=1.871	*U*=3.5	*U*=12	*U*=11
SL/SVL	15.3−17.5	15.4−18.0	14.5−16.5	*p*=0.392$_{ns}$	*p*=0.373$_{ns}$	*p*=0.611$_{ns}$	*p*=0.202$_{ns}$
	23.4±1.1	24.8±0.4	22.6±0.8	x^2=9.466	*U*=0	*U*=8.5	*U*=0
NTL/SVL	22.1−24.0	24.5−25.4	21.5−24.0	*p*=0.009*	*p*=0.032*	*p*=0.267$_{ns}$	*p*=0.005*
	9.6±0.8	9.6±0.7	9.1±0.8	x^2=1.758	*U*=6	*U*=10	*U*=11.5
EN/SVL	8.9−10.6	8.8−10.3	7.4−10.2	*p*=0.415$_{ns}$	*p*=1.000$_{ns}$	*p*=0.396$_{ns}$	*p*=0.228$_{ns}$
	4.5±0.9	4.0±0.2	4.2±0.6	x^2=0.830	*U*=4	*U*=12	*U*=15
TEL/SVL	3.8−5.4	3.6−4.2	3.5−5.0	*p*=0.660$_{ns}$	*p*=0.480$_{ns}$	*p*=0.611$_{ns}$	*p*=0.476$_{ns}$
	7.5±0.4	7.3±0.3	8.7±1.1	x^2=8.329	*U*=3	*U*=3	*U*=3
TD/SVL	7.2−7.9	7.1−7.8	7.4−10.2	*p*=0.016*	*p*=0.266$_{ns}$	*p*=0.042*	*p*=0.016*
	39.0±0.4	36.2±1.5	35.7±1.8	x^2=6.797	*U*=0	*U*=1	*U*=14
MN/SVL	38.5−39.3	35.0−38.1	33.6−38.9	*p*=0.033*	*p*=0.034*	*p*=0.018*	*p*=0.396$_{ns}$
	31.5±1.6	26.3±1.4	30.2±1.8	x^2=7.807	*U*=0	*U*=8	*U*=3
MFE/SVL	30.5−33.3	24.3−27.4	26.2−32.6	*p*=0.020*	*p*=0.034*	*p*=0.235$_{ns}$	*p*=0.016*
	20.8±1.37	16.1±0.3	21.9±2.2	x^2=8.950	*U*=0	*U*=11	*U*=0
MBE/SVL	19.3−22.0	15.8−16.5	19.2−25.0	*p*=0.011*	*p*=0.034*	*p*=0.498$_{ns}$	*p*=0.005*
	8.1±0.1	8.4±0.4	8.1±0.6	x^2=1.915	*U*=2.5	*U*=13.5	*U*=11.5
IN/SVL	7.9−8.2	7.9−8.8	7.0−9.2	*p*=0.384$_{ns}$	*p*=0.208$_{ns}$	*p*=0.796$_{ns}$	*p*=0.224$_{ns}$
	13.2±1.3	12.9±0.3	13.3±0.4	x^2=2.370	*U*=4.5	*U*=10.5	*U*=9
EL/SVL	12.3−14.6	12.6−13.3	12.6−13.8	*p*=0.306$_{ns}$	*p*=0.593$_{ns}$	*p*=0.433$_{ns}$	*p*=0.111$_{ns}$
	8.0±0.4	6.3±0.1	7.1±0.9	x^2=4.858	*U*=0	*U*=5.5	*U*=13
IOD/SVL	7.7−8.4	6.3−6.4	5.8−8.2	*p*=0.088$_{ns}$	*p*=0.031*	*p*=0.108$_{ns}$	*p*=0.320$_{ns}$
	13.9±1.1	13.9±1.0	14.7±0.4	x^2=2.931	*U*=5	*U*=8.5	*U*=9
UEW/SVL	13.2−15.1	12.9−15.3	14.2−15.4	*p*=0.231	*p*=0.724$_{ns}$	*p*=0.269$_{ns}$	*p*=0.119$_{ns}$
	23.5±1.5	22.6±.3	20.8±1.4	x^2=8.612	*U*=4	*U*=3	*U*=2
HAL/SVL	21.7−24.5	22.3−23.0	18.6−22.5	*p*=0.013*	*p*=0.476$_{ns}$	*p*=0.043*	*p*=0.011*
	23.1±0.7	22.9±0.7	20.9±1.3	x^2=9.187	*U*=5	*U*=2	*U*=2
FAL/SVL	22.3−23.6	22.1−23.8	18.6−22.6	*p*=0.010*	*p*=0.724$_{ns}$	*p*=0.028*	*p*=0.011*
	25.7±3.3	30.2±0.5	29.5±1.4	x^2=6.448	*U*=0	*U*=2	*U*=13.5
LAL/SVL	22.0−28.5	29.8−30.8	26.2−31.4	*p*=0.040*	*p*=0.032*	*p*=0.028*	*p*=0.356$_{ns}$
	162.0±8.8	174.1±4.8	139.1±6.5	x^2=12.254	*U*=1	*U*=0	*U*=0
HLL/SVL	154.1−171.5	167.7−178.1	126.2−149.6	*p*=0.002*	*p*=0.077$_{ns}$	*p*=0.011*	*p*=0.005*
	48.9±3.9	48.6±1.2	43.1±2.0	x^2=9.754	*U*=4	*U*=3	*U*=0
THIGHL/SVL	44.4−51.5	47.3−50.2	39.2−45.9	*p*=0.008*	*p*=0.480$_{ns}$	*p*=0.043*	*p*=0.005*
	56.3±2.7	56.8±2.3	46.0±3.0	x^2=11.690	*U*=5	*U*=0	*U*=0
TL/SVL	53.9−59.2	54.6−59.5	39.8−50.6	*p*=0.003*	*p*=0.724$_{ns}$	*p*=0.011*	*p*=0.005*
	53.4±3.5	56.2±1.7	46.2±2.8	x^2=11.873	*U*=3	*U*=0	*U*=0
FOL/SVL	50.2−57.1	54.2−58.3	41.4−50.1	*p*=0.003*	*p*=0.289$_{ns}$	*p*=0.011*	*p*=0.005*
	77.4±6.8	81.1±4.0	66.1±4.2	x^2=11.066	*U*=5	*U*=1	*U*=0
TFOL/SVL	71.2−84.7	75.2−84.0	59.9−73.3	*p*=0.004*	*p*=0.724$_{ns}$	*p*=0.018*	*p*=0.005*
	12.0±0.6	12.4±0.3	11.4±0.7	x^2=5.575	*U*=3	*U*=9	*U*=4
3FL/SVL	11.5−12.6	11.9−12.7	10.0−12.5	*p*=0.062$_{ns}$	*p*=0.289$_{ns}$	*p*=0.310$_{ns}$	*p*=0.023*
	13.0±1.3	11.5±0.5	12.2±0.8	x^2=5.357	*U*=1	*U*=7.5	*U*=7
1FL/SVL	11.9−14.4	11.0−12.1	11.1−13.5	*p*=0.069$_{ns}$	*p*=0.075$_{ns}$	*p*=0.204$_{ns}$	*p*=0.065$_{ns}$
	32.8±0.9	33.7±1.2	27.6±2.4	x^2=11.239	*U*=3.5	*U*=1	*U*=0
4TL/SVL	31.8−33.6	32.5−35.3	24.3−31.9	*p*=0.004*	*p*=0.373$_{ns}$	*p*=0.018*	*p*=0.005*
	7.0±1.0	5.6±0.6	6.2±0.5	x^2=4.831	*U*=2	*U*=7	*U*=7
IMTL/SVL	5.9−7.9	4.8−6.1	5.2−6.8	*p*=0.089$_{ns}$	*p*=0.157$_{ns}$	*p*=0.175$_{ns}$	*p*=0.065$_{ns}$
	12.0±1.6	17.9±0.6	10.3±0.7	x^2=10.789	*U*=0	*U*=4	*U*=0
ITL/SVL	10.4−13.7	17.2−18.6	7.0−11.3	*p*=0.005*	*p*=0.034*	*p*=0.061$_{ns}$	*p*=0.005*

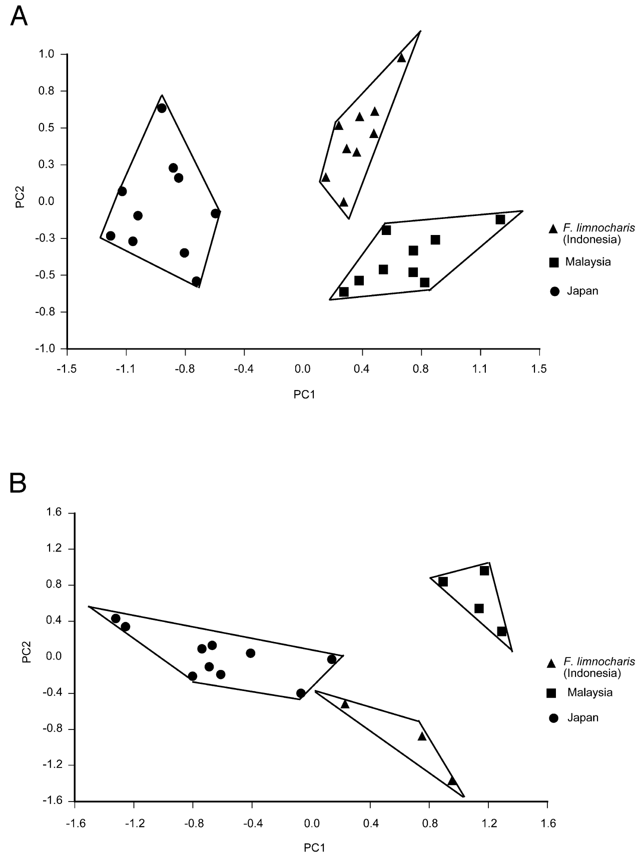

Fig. 2. Plot of principal component 1 (PC1) versus principal component 2 (PC2) for the principal component analysis of the *F. limnocharis* group. (A) Males. (B) Females.

tions, 10.2% contained 13 bivalents and 89.8% contained 2–16 univalents. Among them, 21.4% contained 2 univalents, 21.2% contained 4 univalents, and 19.6% contained 6 univalents, with the mean number per cell 5.27. In hybrids between Japan and Malaysia populations, 12.4% contained 13 bivalents, and 87.6% contained 2–14 univalents. Among them, 29.2% contained 4 univalents, 20.8% contained 2 univalents, and 15.4% contained 6 univalents, with the mean number per cell 4.58. In hybrids between Malaysia and Indonesia populations, 80.4% contained 13 bivalents and 19.6% contained 2–6 univalents, with the mean number per cell 0.48.

The numbers of ring- and rod-shaped bivalents in the three populations and in the hybrids are shown in Table 6. In the control Japan and Malaysia populations, the frequency of ring-shaped bivalents was 99.7% and 99.6%, respectively, with the mean number per cell 13. In hybrids between the Japan and Indonesia populations, the Japan and Malaysia populations, and the Malaysia and Indonesia populations, the frequency of ring-shaped bivalents was 36%, 36%, and 59.5% with the mean number per cell 10.47, 10.74, and 12.70, respectively.

Table 4. Factor loading on the first two principal component analyses extracted from the correlation matrix of 31 characters for male and female of the *F. limnocharis* group.

	Male		female	
Characters	PC1	PC2	PC1	PC2
SVL	0.06	0.09	0.03	0.06
HL	0.08	0.34	0.13	–0.35
HW	–0.14	0.30	–0.07	–0.34
STL	0.19	0.15	0.24	–0.07
MSL	0.13	0.25	0.23	–0.12
NS	–0.12	0.13	–0.16	–0.27
SL	0.18	–0.13	0.14	–0.05
NTL	0.22	0.01	0.23	0.08
EN	0.24	–0.01	0.12	–0.03
TEL	–0.02	–0.16	0.01	–0.14
TD	–0.21	0.09	–0.19	0.04
MN	0.12	0.31	0.08	–0.24
MFE	–0.10	0.38	–0.15	–0.30
MBE	–0.19	0.24	–0.20	–0.21
IN	–0.13	0.12	0.14	0.03
EL	–0.13	0.12	–0.02	–0.18
IOD	0.15	0.25	0.03	–0.30
UEW	–0.17	0.14	–0.10	–0.07
HAL	0.24	0.09	0.22	–0.15
FAL	0.21	0.08	0.22	–0.07
LAL	0.00	–0.14	–0.01	0.19
HLL	0.26	–0.03	0.27	0.01
THIGHL	0.24	0.09	0.25	–0.08
TL	0.25	0.07	0.27	–0.06
FOL	0.23	0.01	0.27	–0.01
TFOL	0.25	0.01	0.27	–0.02
3FL	0.21	0.13	0.21	0.03
1FL	0.17	0.20	0.00	–0.27
4TL	0.25	–0.03	0.27	–0.02
IMTL	–0.06	0.28	0.01	–0.36
ITL	0.19	–0.22	0.23	0.20
Eigenvalues	14.37	5.11	12.67	5.49
Variance explained (%)	46.35	16.47	40.86	17.69
Cumulative explained (%)	46.35	62.82	40.86	58.55

Sequence divergence and phylogeny

Nucleotide sequence data comprising 499-bp and 591-bp segments of the 16S rRNA and Cyt *b* genes, respectively, were used for analyses. The 16S alignment contained 112 polymorphic sites, of which 20 were parsimony-informative, and the Cyt *b* alignment contained 200 polymorphic sites, of which 137 were parsimony-informative. Tables 7 and 8 show the sequence divergence among 16S and Cyt *b* haplotypes in the *F. limnocharis* group (including *F. multistriata*), *F. iskandari*, and the outgroup (*Limnonectes fujianensis*). Sequence divergences were smaller in 16S than in Cyt *b*. In 16S rRNA, sequence divergence was 17.0–18.1% ($\bar{x}$ =17.2%) between the outgroup (*L. fujianensis*) and the *F. limnocharis* group and *F. iskandari*; 10.6–11.0% ($\bar{x}$=10.9%) between *F. iskandari* and the *F. limnocharis* group; 2.4–2.6% ($\bar{x}$=2.52%) between the Japan population and another *F. limnocharis* group; 0.2–0.4% ($\bar{x}$=0.3%) between *F. multistriata* and the Malaysia and Indonesia populations; and 0.0–0.4% ($\bar{x}$=0.23%) among the Malaysia and Indonesia populations. Sequence divergence in the Cyt *b* gene for

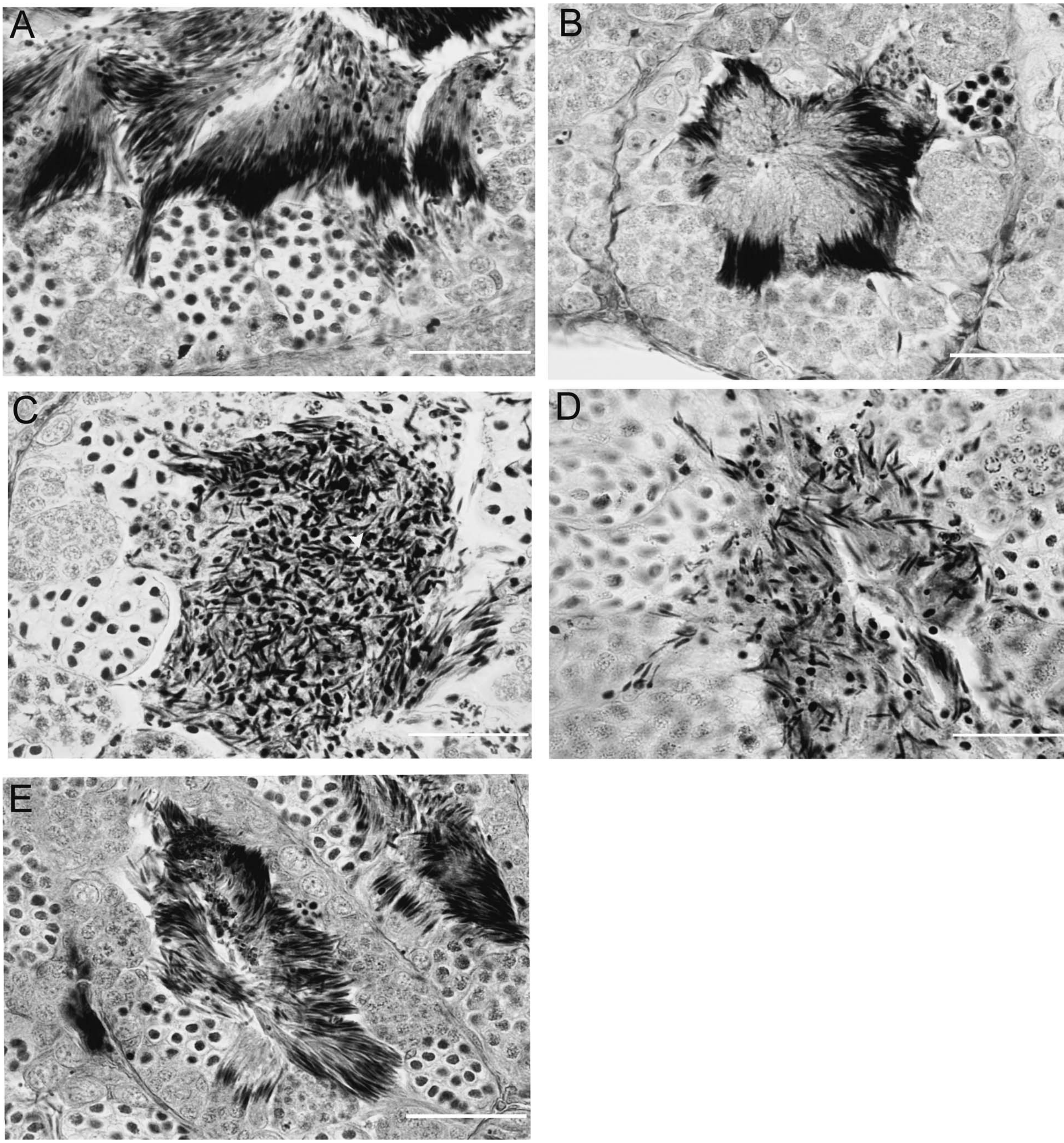

Fig. 3. Cross-sections of seminiferous tubules in the testes of controls and of hybrids among three populations of the *F. limnocharis* group. Scale bars, 20 μm. **(A)** Control Japan population. **(B)** Control Malaysia population. **(C)** Hybrid between Japan female and *F. limnocharis* (Indonesia) male. **(D)** Hybrid between Japan female and Malaysia male. **(E)** Hybrid between Malaysia female and *F. limnocharis* (Indonesia) male.

each of the above combinations was 20.6–23.7% ($\bar{x}$ =21.5%); 18.3–20.6% ($\bar{x}$=19.0%); 11–12% ($\bar{x}$=11.46%); 0.3–1.2% ($\bar{x}$=0.8%); and 0.3–1.5% ($\bar{x}$=1.0%).

The ML tree based on the 16S rRNA gene sequences showed that the Malaysia, and Indonesia populations and *F. multistriata* from China made up one cluster, with strong bootstrap support (BP) of 97, 99, and 100% in the ML, MP and NJ trees (Fig. 6), respectively. However, the Japan population was separate, with 100% BP in the ML, MP and NJ trees. The same result was also obtained for Cyt *b*, with BP of 96, 100, and 100% in the ML, MP, and NJ trees (Fig. 7). The Indonesia population was less differentiated from the Malaysia population and *F. multistriata* from China, with BP of 60, 81, and 93% in the ML, MP, and NJ trees, and *F. multistriata* from China formed a clade with the Malaysia population.

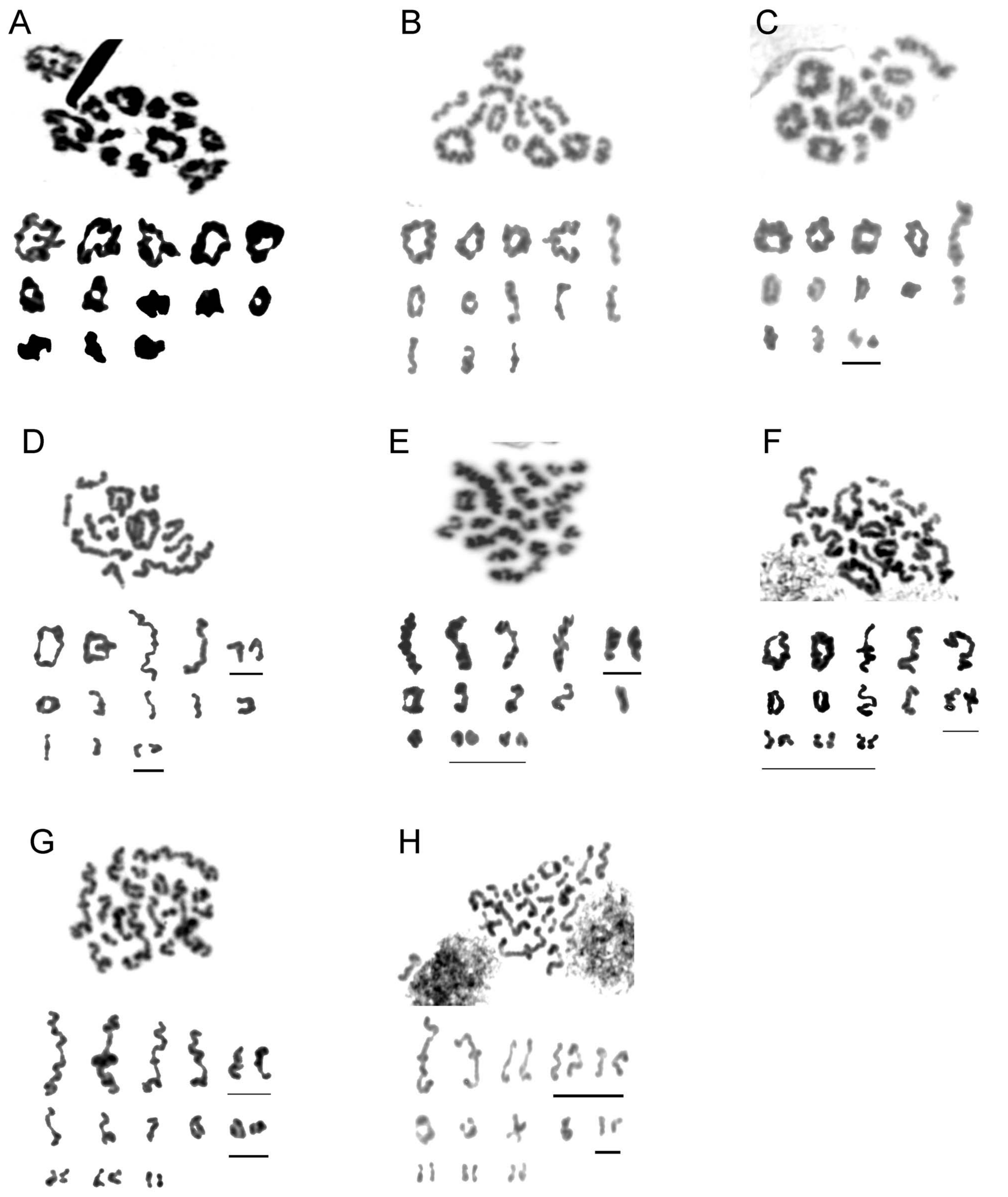

Fig. 4. Spermatocytes at the first meiosis and chromosome complements in controls and in hybrids among three populations of the *F. limnocharis* group. Bars under the chromosomes indicate univalents. **(A)** Control Japan population containing 13 bivalents, all of them ring-shaped. **(B)** Hybrids containing 13 bivalents, and ring- or rod-shaped chromosomes. **(C–G)** Hybrids contained 2–10 univalents. Hybrids containing 14 univalents.

Table 5. Numbers of meiotic spreads differing in number of univalents in male hybrids among three populations of the *F. limnocharis* group and the controls.

Type of frogs	No. of meioses	No. of univalents (%)									Mean no. of univalents
		0	2	4	6	8	10	12	14	16	per cell
Japan control	323	323 (100)									0
Malaysia control	166	166 (100)									0
Japan x *F. limnocharis*	394	39 (10.2)	82 (21.4)	81 (21.2)	75 (19.6)	53 (13.8)	39 (10.2)	14 (3.7)	10 (2.6)	1 (0.3)	5.27
Japan x Malaysia	332	41 (12.4)	69 (20.8)	97 (29.2)	51 (15.4)	38 (11.5)	27 (8.1)	6 (1.8)	3 (0.9)		4.58
Malaysia x *F. limnocharis*	311	250 (80.4)	51 (16.4)	7 (2.3)	3 (1.0)						0.48

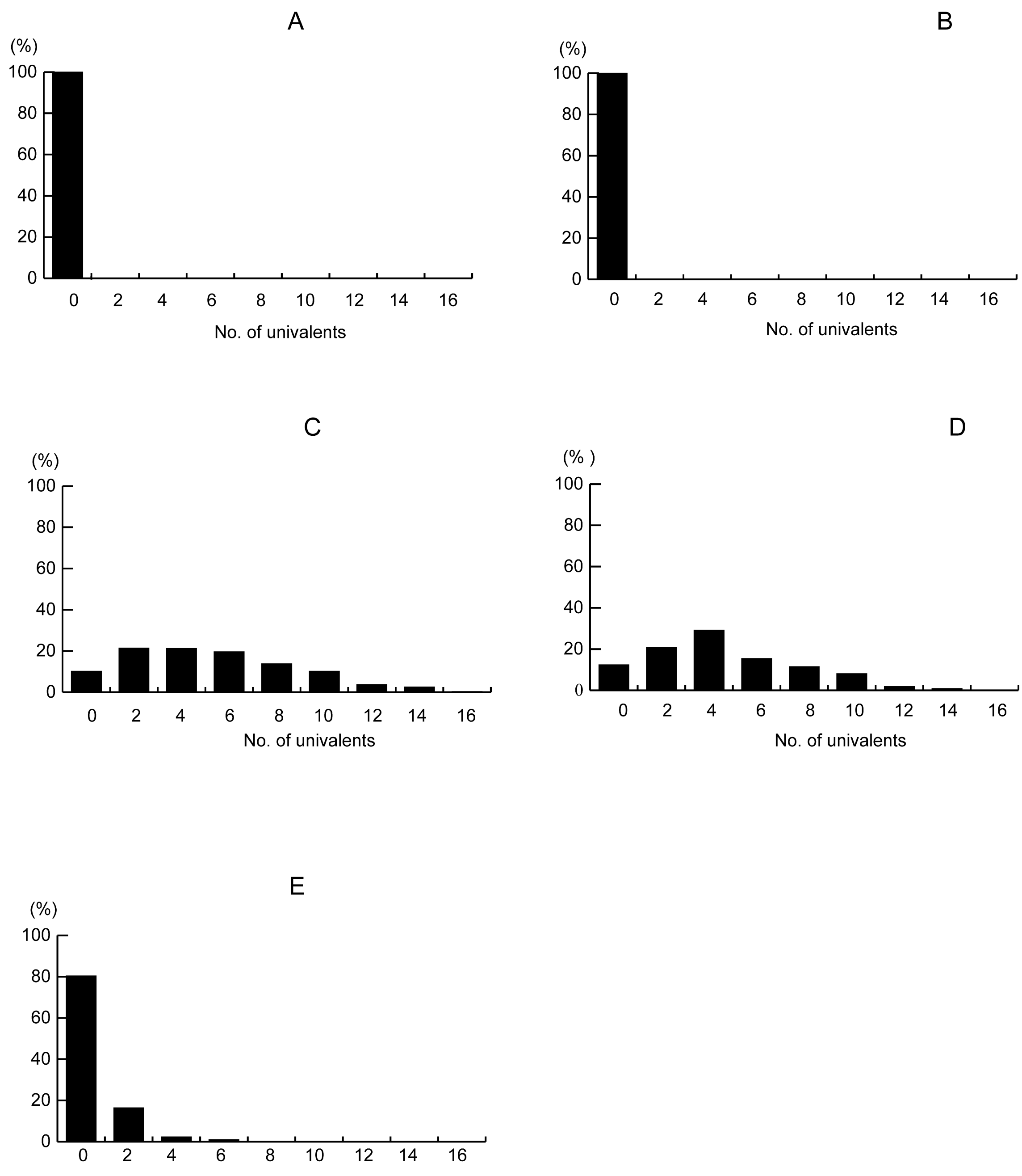

Fig. 5. Frequency distributions of univalents in the meiotic spreads of male hybrids among controls and among three populations of the *F. limnocharis* group. **(A)** Control Japan population. **(B)** Control Malaysia population. **(C)** Hybrid between Japan female and *F. limnocharis* (Indonesia) male. **(D)** Hybrid between Japan female and Malaysia male. **(E)** Hybrid between Malaysia female and *F. limnocharis* (Indonesia) male.

Table 6. Numbers of the ring- and rod-shaped bivalents in male hybrids among three populations of the *F. limnocharis* group and the controls.

Type of frog	No. of bivalents	Large chromosome		Small chromosome		Total		Mean no. of
		Ring (%)	Rod (%)	Ring (%)	Rod (%)	Ring (%)	Rod (%)	bivalents per cell
Japan	4199	1610 (99.6)	5 (0.2)	2577 (99.7)	7 (0.2)	4187 (99.7)	12 (0.3)	13
Malaysia	2158	826 (99.5)	4 (0.3)	1324 (99.7)	4 (0.2)	2150 (99.6)	8 (0.4)	13
Japan *x F. limnocharis*	4577	810 (48.3)	866 (51.7)	840 (29.0)	2061 (71.0)	1650 (36.0)	2927 (64.0)	10.47
Japan x Malaysia	3566	517 (39.1)	804 (60.9)	768 (34.2)	1477 (65.8)	1285 (36.0)	2281 (64.0)	10.74
Malaysia x *F. limnocharis*	3949	1309 (87.3)	191 (12.7)	1040 (42.5)	1409 (57.5)	2349 (59.5)	1600 (40.5)	12.70

Table 7. Percent sequence divergences based on the uncorrected p-distances among haplotypes of 16S rRNA gene sequences in the *F. limnocharis* complex from several Asian countries.

	Kual-1	Kual-2	Kota-1	Kota-2	Mali (lim)	Bogo (lim)	Hai (mul)	Hiro	Cian (isk)	China (Limno)
Kuala Lumpur-1 (Malaysia)	–									
Kuala Lumpur-2 (Malaysia)	0.4	–								
Kota Kinabalu-1(Sabah, Malaysia)	0.2	0.2	–							
Kota Kinabalu-2 (Sabah, Malaysia)	0.2	0.2	0	–						
F. limnocharis (Malingping, Java, Indonesia)	0.4	0.4	0.2	0	–					
F. limnocharis (Bogor, Java, Indonesia)	0.2	0.2	0	0.2	0.2	–				
F. multistriata (Hainan, China)	0.4	0.4	0.2	0.2	0.4	0.2	–			
Higashihiroshima (Japan)	2.6	2.6	2.4	2.4	2.6	2.4	2.6	–		
F. iskandari (Cianjur, Java, Indonesia)	10.4	11.0	10.8	10.8	11.0	10.8	11.0	10.6	–	
Limnonectes fujianensis (China)	17.2	17.2	17.0	17.0	17.2	17.0	17.0	17.6	18.1	–

Table 8. Percent sequence divergences based on the uncorrected p-distances among haplotypes of Cyt *b* gene sequences in the *F. limnocharis* complex from several Asian countries.

	Kual-1	Kual-2	Kual-3	Kota-1	Kota-2	Bogo (lim)	Mali (lim)	Hain (mul)	Higa	Hiro	Cian (isk)	Mali (isk)	China (Limno)
Kuala Lumpur-1 (Malaysia)	–												
Kuala Lumpur-2 (Malaysia)	0.5	–											
Kuala Lumpur-3 (Malaysia)	0.7	0.5	–										
Kota Kinabalu-1 (Sabah, Malaysia)	0.7	0.7	1.0	–									
Kota Kinabalu-2 (Sabah, Malaysia)	0.7	1.2	1.4	1.0	–								
F. limnocharis (Bogor, Java, Indonesia)	1.0	1.2	1.4	1.0	1.4	–							
F. limnocharis (Malingping, Java, Indonesia)	1.2	1.4	1.5	1.2	1.5	0.9	–						
F. multistriata (Hainan, China)	0.3	0.9	1.0	0.7	0.7	1.0	1.2	–					
Higashihiroshima(Japan)	11.7	11.3	11.7	11.5	11.8	11.8	12.0	11.7	–				
Hiroshima (Japan)	11.0	12.0	11.0	11.2	11.2	11.2	11.3	11.0	2.0	–			
F. iskandari (Cianjur, Java, Indonesia)	18.6	19.0	19.1	18.8	19.0	18.8	18.8	18.4	20.6	19.8	–		
F. iskandari (Malingping, Java, Indonesia)	18.4	18.8	19.0	18.6	18.8	18.6	18.6	18.3	20.5	19.6	0.2	–	
Limnonectes fujianensis (China)	20.6	20.8	20.0	20.6	20.0	20.0	10.8	20.6	23.7	22.8	22.2	22.2	–

DISCUSSION

Taxonomic status of the Malaysia population

Toda *et al.* (1998) first recognized two syntopically occurring species within the *F. limnocharis* complex in Java, Indonesia. On the basis of allozyme data, they further suggested the presence of at least four species, including the two above, within the *F. limnocharis* complex from Indonesia, Laos, Hong Kong, and China. However, Toda *et al.* (1998) reserved taxonomic decisions on these taxa, because they thought it premature to do so without examining sufficient samples of the *F. limnocharis* complex to cover its wide distribution. Later, Veith *et al*. (2001) similarly recognized two cryptic species in the *F. limnocharis* complex occurring in sympatry in Java. These two species show substantial genetic differentiation, but are morphologically hardly distinguishable from one another. They applied the name *F. limnocharis* (Gravenhorst) to the taxon widely distributed in Java, Sumatra and Borneo, and described the taxon known only from Java as *F. iskandari*. Meanwhile, Dubois and Ohler (2000) designated a neotype for *Rana multistriata* (Hallowell, 1860) from Hong Kong, China. Fei *et al.* (2002) considered this name to be applied to the Chinese rice frog, long treated as *R.* (=*Fejervarya*) *limnocharis* (e.g., Peters, 1863), and used the name *F. multistriata* for the frogs from all over China and Taiwan (Fei *et al*., 2005). Similarly, the Malaysia population has been treated as *R. limnocharis* (e.g., Boulenger, 1912; Smith, 1930: Berry, 1975; Inger and Voris, 2001). However, on the basis of allozyme analyses and crossing experiments, Djong *et al*. (2007) suggested that *F. limnocharis* from Malaysia may differ from the topotypic *F. limnocharis* at the subspecies or species level.

Morphological differentiation of amphibian taxa of subspecies and even specific rank is often very small and involves mainly difference in body proportions (Babik and Rafinski, 2000). The present analyses showed that nine and ten morphological characteristics differed between the Malaysia population and *F. limnocharis* in males and females, respectively. The main difference was in the head shape. The head of *F. limnocharis* was longer and broader

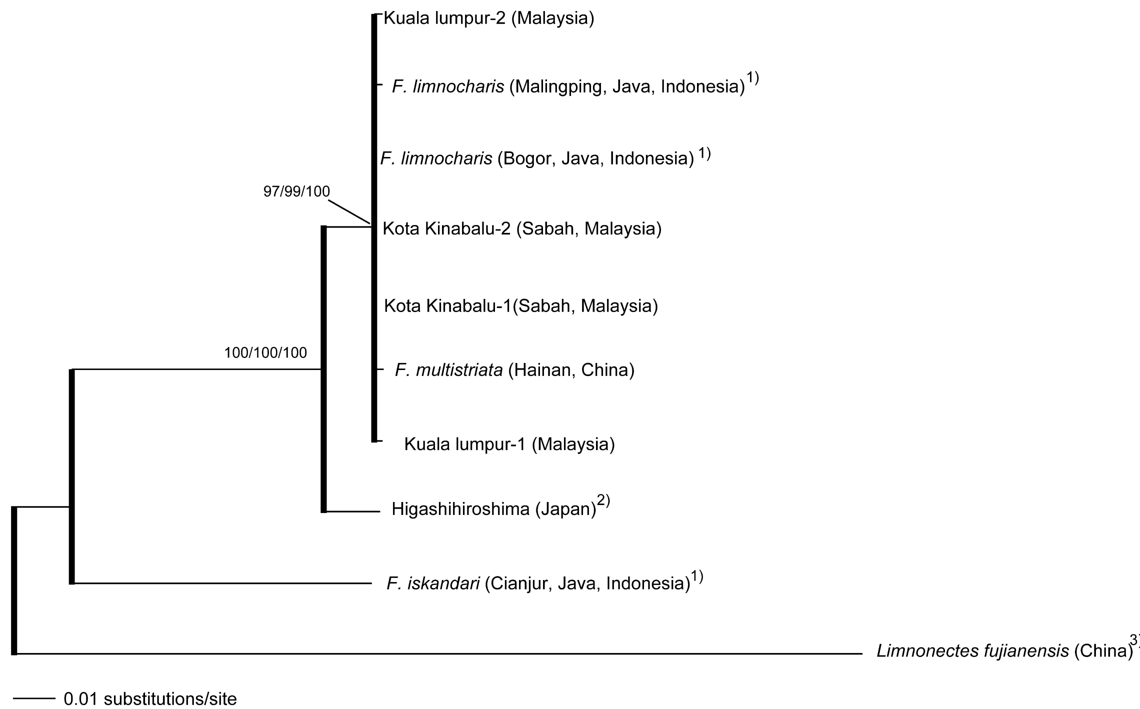

Fig. 6. Phylogenetic tree constructed by the maximum likelihood (ML) method for the *F. limnocharis* group based on nucleotide sequences of the 16S rRNA gene. The numbers at each node indicate BP values calculated by MP/ML/NJ. BP values were calculated based on 1,000 replicates. The scale bar represents branch length in nucleotide substitutions per site. [1] Kotaki *et al.* (2008). [2] Sumida *et al.* (2002). [3] Nie *et al*. (unpublished).

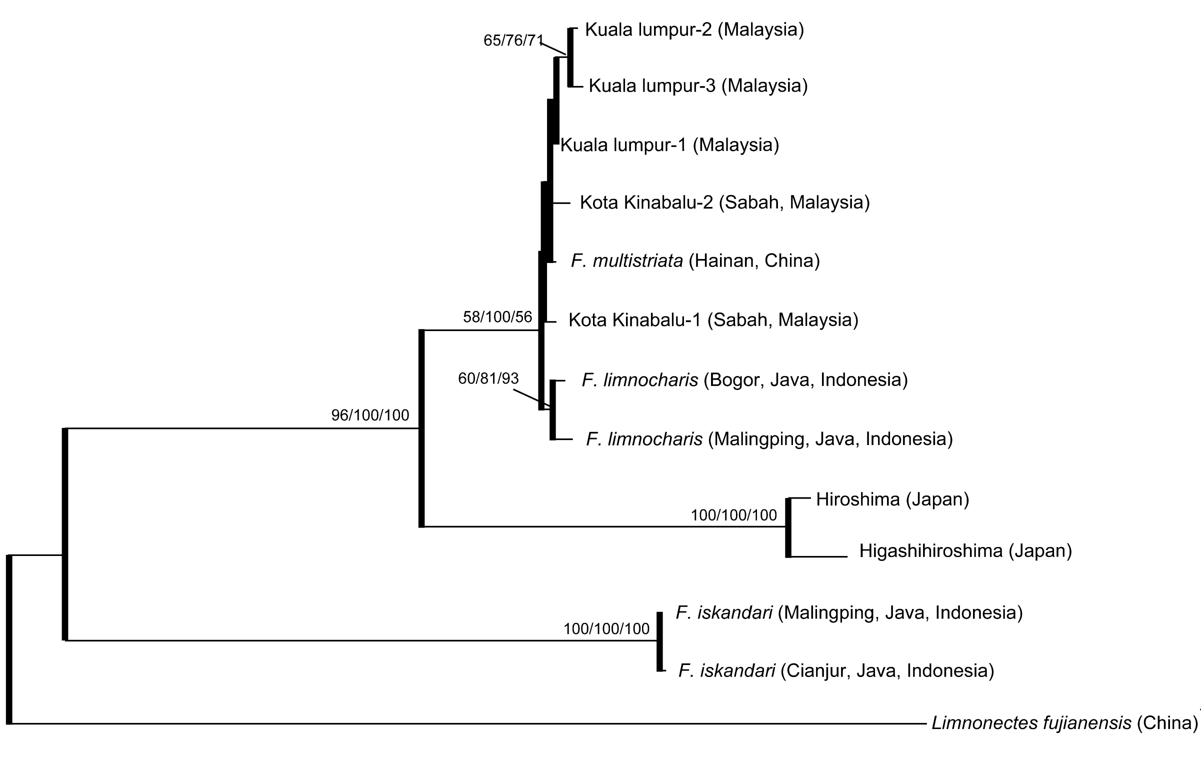

Fig. 7. Phylogenetic tree constructed by maximum likelihood (ML) method for the *F. limnocharis* group based on nucleotide sequences of Cyt *b* gene. The numbers at each node indicate BP values calculated by MP/ML/NJ. BP values were calculated based on 1,000 replicates. The scale bar represents branch length in nucleotide substitutions per site. [1] Nie *et al*. (unpublished).

than that of the Malaysia population. Veith *et al*. (2001) likewise found that *F limnocharis* from Sumatra, Borneo, and Java was significantly different in head shape. Our present morphological data subjected to cluster and principal component analyses indicated that the Malaysia population could be reasonably regarded as a subspecies of *F. limnocharis*.

Histological observation on the testes of hybrids between Malaysia and Indonesia populations showed almost the normal condition, filled with dense bundles of normal spermatozoa. Sumida *et al.* (2002) found that in hybrids between the Sakishima Island (Ishigaki and Iriomote) populations and main-island Japan populations of *F. limnocharis*, the testes were almost normal in inner structure, and regarded these populations as subspecies. Spermatogenesis also showed some abnormality: 80.4% contained 13 bivalents and 19.6% contained 2–6 univalents, with the mean number of univalents per spermatocyte 0.48 and the frequency of ring-shaped and rod-shaped bivalents 59.5% and 40.5%, respectively. Callan and Spurway (1951) found that hybrids between European newts *Triturus cristatus carnifex* (=*T. carnifex*) and *T. c. karelinii* (=*T. karelinii*) had 0.9–4.3 (mean 2.44) univalents per spermatocyte and drastic reduction in chiasma frequency, with most of the chiasma forming terminals, and they regarded these taxa as subspecies. The current taxonomic status of these distinct species is considered to be correct on the basis of the degree of abnormality in spermatogenesis. Sumida *et al.* (unpublished) also found that the testes in hybrids between Sakishima Island (Ishigaki and Iriomote) and main-island Japan populations showed some abnormal spermatogenesis: 65.9% contained 13 bivalents and 34.1% contained 2–6 univalents, with the mean number of univalents per spermatocyte 0.88 and the frequency of ring-shaped and rod-shaped bivalents 84.9% and 15.1%, respectively. Thus, it is not unreasonable to regard the Malaysia populations as a subspecies of *F. limnocharis.*

The mean sequence divergence of 16S rRNA and *Cyt b* was 0.2% and 1.3%, respectively, between Malaysia and Indonesia populations of *F. limnocharis*. Several previous studies on sequence divergence among amphibian populations have used 16S and Cyt *b* sequences. 16S sequence divergence was 0.7–1.5% among *Mantidactylus granulatus* populations (Vences *et al.,* 2003). Vences *et al.* (2004b) mentioned that differentiation among conspecific populations never exceeds 2% for the 16S rRNA gene. Sequence divergence of Cyt *b* was 0.2–2.1% among populations and 3.7–4.6% among subspecies in Japanese pond frogs (Sumida *et al.,* 1998). Our present data suggested that Malaysia populations of *F. limnocharis* complex are one species. Our ML trees showed that the Malaysia and Indonesia populations diverged in Cyt*,* but not in 16S. On the other hand, Djong *et al*. (2007) showed that the mean Nei's (1972) genetic distance between Malaysia and Indonesia populations was 0.451 (range 0.410–0.526). This genetic distance could be regarded as delimiting either subspecies or species based on Thorpe (1982), Highton (1989), and Skibinski *et al.* (1993), who reported that genetic distances above 0.15 can be considered to indicate different species. Nishioka and Sumida (1990) also viewed genetic distances above 0.301 as the borderline between species and subspecies. Thus, the previous allozyme data also showed clear differentiation between Malaysia and Indonesia populations probably at above the subspecies level.

The mean sequence divergence between topotypic *F. limnocharis* and Chinese *F. multistriata* was 0.2–0.4% (mean 0.3%) and 1.0–1.2% (mean 1.1%) for 16S and Cyt *b*, respectively. Although the 16S ML tree showed no differentiation among topotypic *F. limnocharis*, Chinese *F. multistriata,* and Malaysia populations, the Cyt *b* ML tree showed slight differentiation between topotypic *F. limnocharis* and Chinese *F. multistriata*. Based on these results, it is reasonable to regard *F. multistriata* as a subspecies of *F. limnocharis*, although further examination will be necessary for clarifying the taxonomic status of this species.

The concept of subspecies has been used at least since Linnaeus' time. Charles Darwin proffered qualitative definitions and considered varieties to be incipient species, potentially evolving into full species. Mayr (1963) defined subspecies as geographically defined aggregates of local populations that differ taxonomically from other such subdivisions of the species, ordinarily under conditions of allopatry (reproductive barriers are geographic). Traditionally, subspecies have been defined by morphological traits or color variations, but recent critics have been concerned that these traits may not reflect underlying genetic structure and phylogeny (Ball and Avise, 1992). Therefore, it is important to provide formal criteria for subspecies classification. O'Brien and Mayr (1991) offered several guidelines: members of a subspecies share a unique geographic range or habitat, a group of phylogenetically concordant phenotypic characteristics, and a unique natural history relative to other subdivisions of the species; they are below the species level, and different subspecies are reproductively compatible. Sumida (1994) maintained that in cytogenetic studies of meiosis in F1 hybrids between closely related species or subspecies, the one important criterion by which to conclude species or subspecies status is chromosome behavior at spermatogenesis. Avise (2000) suggested that analysis of mitochondrial DNA (mtDNA) sequence variation within and among subspecies reveals whether subspecies are evolving independently, are freely exchanging breeding individuals, or are at some intermediate stage of isolation.

In the present study, the morphological data, backcrossing experiments, and histological and spermatogenic observations of the testes of the hybrids between *F. limnocharis* and Malaysia populations and the molecular phylogenetic relationship based on the Cyt *b* gene data suggest that the Malaysia population be regarded as a subspecies of *F. limnocharis*. Further examination is necessary to accurately elucidate the status of this population.

Taxonomic status of the Japan population

The rice frog from Japan was treated as *Rana limnocharis* (=*Fejervarya limnocharis*) (Stejneger, 1907; Okada, 1931; Nakamura and Ueno, 1963). These authors designated this name for populations distributed on the main islands, Honshu from the Chubu district and westwards, Shikoku, and Kyushu, and the southwestern islands of Japan. Kuramoto (1973, 1979) and Ota (1981, 1983) carried out a series of studies on morphological variation in the Japan populations of this species, and Nishioka and Sumida

(1990) and Toda *et al.* (1997, 1998) studied genetic variation. The taxonomic status of the populations from the Sakishima Islands was still under debate. Maeda and Matsui (1989) concluded on the basis of external morphology, mating calls, and genetic distances that the Sakishima Island populations were differentiated as a species distinct from the main-island populations. On the other hand, Sumida *et al*. (2002) inferred from the crossing experiments and molecular data that it is reasonable to regard the Sakishima Island populations as a subspecies.

Our morphological data based on the cluster and principal component analyses showed that the Japan population is considerably differentiated from the Malaysia and Indonesia populations. The main different characteristics were head length, tympanum diameter, and forelimb and hindlimb length. The head in the Japan population was shorter than that of the Indonesia population. Tympanum diameter in the Japan population was larger than that in the Indonesia population. The forelimb of the Japan population was shorter than that of the Indonesia population, especially in hand length and forearm length. The hindlimb of the Japan population was also shorter than that of the Indonesia population in hindlimb length, thigh length, tibia length, and foot length. These data strongly suggest that the Japan population is morphologically differentiated from Indonesia *F. limnocharis* as a distinct species. Emerson (1986) mentioned that differences in relative hindlimb length might be the result of unequal growth and developmental rate during the larval period. Wilbur and Collins (1973) suggested that one of the main factors influencing the length of the amphibian larval period is temperature. Blouin and Brown (2000) showed that temperature-induced variation in the growth rate of tadpoles of *Rana cascadae* caused some variation in head width and leg length at metamorphosis. Babik and Rafinski (2000) showed that differences in water temperature during the larval period may be responsible for variation in hindlimb length in Central European *Rana arvalis*, as indicated by the generally shorter legs in Polish specimens correlating with the cooler climate of the northern area. Ishchenko (1977) also showed an altitudinal cline in body proportions in *R. macrocnemis*, in which frogs from higher altitudes were relatively short legged. These observations indicate that temperature may be the most important factor influencing relative hindlimb length in frogs.

Histological and spermatogenic observations showed some abnormalities in hybrids between the Indonesia and Japan populations and also between the Malaysia and Japan populations. In the seminiferous tubules of the testes, there were considerably abnormal spermatozoa (the sperm head was larger than that of normal spermatozoa) and pycnotic nuclei, as reported in several interspecific hybrids (Ueda, 1977; Kawamura *et al.,* 1980; Kuramoto, 1983; Sumida *et al.*, 2003). Spermatogenic observation also showed considerable abnormality in meiosis in these hybrids. In the hybrids between the Japan and Indonesia populations, 10.2% of meiotic spreads contained 13 bivalents and 89.8% of meiotic spreads contained 2–16 univalents, with the mean number per spermatocyte 5.27. In hybrids between the Japan and Malaysia populations, 12.4% contained 13 bivalents and 87.6% contained 2–14 univalents, with the mean number per spermatocyte 4.58. White (1946) also observed abnormal meioses with an increase in the number and frequency of univalents in hybrids between *Triturus marmoratus* and *T. cristatus carnifex* (=*T. carnifex*), and Spurway and Callan (1960) in hybrids between *T. vulgaris* and *T. helveticus*, with a mean univalent frequency per spermatocyte of 11.3±0.2. Mancino *et al.* (1978) found that in hybrids between *T. cristatus carnifex* (=*T. carnifex*) and *T. vulgaris meridionales*, most of the primary spermatocytes contained only univalents. In hybrids between *T. cristatus* and *T. vulgaris,* the first spermatocytes tended to be asynaptic (Mancino *et al*., 1979). Okumoto (1980) found that in hybrids between female *Rana nigromaculata* and male *R. porosa brevipoda,* the mean number of univalents per spermatocyte was 13.51, and in the reciprocal hybrids, 14.13. These data show that the number of univalents per spermatocyte in the several interspecific hybrids between the Japan and Indonesia or Malaysia populations was smaller than that in the several interspecific hybrids mentioned above. Establishment of reproductive isolation between these populations of the *F. limnocharis* complex is not yet complete and is still in progress.

Molecular analyses of both 16S and Cyt *b* confirmed that the Japan populations comprise a separate cluster from the *F. limnocharis* complex of the Indonesia, Malaysia, and China populations. Sequence divergences between the Japan population and *F. limnocharis* from Indonesia and Malaysia and *F. multistriata* from China were 2.4–2.6% (mean 2.5%) and 11.0–12.0% (mean 11.5%) for 16S and Cyt *b*, respectively. Vences *et al.* (2002) mentioned that sequence divergence in 16S was smaller than 5% in allopatrically separated species. Furthermore, sequence divergence of the Cyt *b* gene was shown to be 10.4–12.4% among Japanese pond frog species (Sumida *et al*., 1998), and above 12.2% among *Discoglossus* species (Zangari *et al*., 2006). Bradley and Baker (2001) mentioned that a sequence divergence in Cyt *b* between 2% and 11% would merit additional study concerning specific status, and that values more than 11% would indicate as recognition of species. After reviewing 24,000 vertebrate and invertebrate species, Kartavtsev and Lee (2006) showed that the average sequence divergence in Cyt *b* among species within a genus is 10.7%±1.3%. Phylogenetic trees constructed by the ML, MP, and NJ methods clearly show that the Japan populations diverged from other populations of the *F. limnocharis* complex in both 16S and Cyt *b*. The data from both these genes data clearly showed that the Japan population is considerably diverged from *F. multistriata* from Hainan, China*.* Thus, we consider that the Japan population forms a lineage separate from both *F. limnocharis* and *F. multistriata,* and can be regarded as an undescribed species; its description is underway (Djong *et al*., in preparation).

The present results also confirm the conclusion by Djong *et al.* (2007) that Japan populations may be a subspecies or distinct species of topotypic *F. limnocharis*. Futhermore, Djong *et al.* found that Nei's (1972) genetic distance between the Indonesia and Japan populations was 0.365–0.638 (mean 0.480). If species delimitation based on genetic distance can be applied as mentioned in the section "Taxonomic status of the Malaysia population," this genetic distance might be regarded as indicating a different species.

Furthermore, although Sasa *et al.* (1998) considered that a genetic distance of 0.3 is the threshold for hybrid inviability, these populations were not isolated by hybrid inviability or hybrid sterility, but only by abnormal spermatogenesis.

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